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Formulation and evaluation of an ointment from *Pinus gerardiana* extracts indigenous to Balochistan

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Abstract: In skin disorders such as microbial and fungal infections, plants and their parts are used. However, there have been very few scientific reports of herbal extracts of the plant *Pinus gerardiana* to be administered transdermally. The antifungal activity was assessed using poisoned food method against the strains of three pathogenic fungi, namely *Alternaria alternata, Curvularia lunata* and *Bipolaris specifera*. Ointment was prepared according to British pharmacopeia and physiochemical evaluation tests were performed. The GCMS was used to determine the chemical composition of the essential oil of *Pinus gerardiana.* 27 components were obtained. Monoterpenes= 89.97%, Oxygenated monoterpenes = 8.75%, Sesquiterpenes = 2.21% out of 100% of the total composition. The extract of *pinus gerardiana* showed a zone of inhibition on organism *Bipolaris specifera* 2.98±0.1μg/ml, *Alternaria alternate* 3.48±0.21μ/ml and *Curvularia lunata* 5.04±0.24μg/ml. Ointment was prepared with pH 5.9, conductivity 0.1, viscosity 22.24 and tested for stability. Franz cells were used in vitro, and release was determined from 30 minutes to 12 hours.

Keywords: Antifungal, Balochistan, ointment, *Pinus gerardiana,*

INTRODUCTION

The skin of the human body acts as a protector against many pathogens. However, sometimes pathogens can disrupt the skin's protective characteristics, causing skin disorders or infections. Infectious agents such as parasites, viruses, bacteria and fungi may cause skin disorders (Imtiaz *et al.,* 2019). Fungal infections are identified by signs such as itchy red spots, hair loss and crusty areas. Wearing tight-fitting clothing or sharing a locker room, clothes, or furnishings with an infected individual are typical causes of fungal infection (Jain *et al.,* 2010). Fungal infections are more dangerous because they originate on the third layer of skin (Shields *et al.,* 2019). Fungi affect keratin-based tissues, including skin, nails, and hair (Abd Elaziz *et al.,* 2020). Sever skin infections can be caused by *Tinea faciei, Tinea pedis, Tinea manuum, Tinea cruris, Alternaria alternata, Curvularia lunata and Bipolaris specifera* fungi (Wijesiri *et al.*, 2018). Fungal infections are being treated by topical, oral and injectable drugs; however, oral and injectable antifungal treatments are more hazardous to the human body than topical. Topical antifungal drugs target fungal infections in many places, but can cause redness, burning and allergic reactions at the application site (Nami *et al.,* 2019). Due to the drug's quick release and poor penetration, it is occasionally necessary to provide therapy for a longer duration. In addition, some drugs may not reach the intended site, which may result in an incomplete clearance of the infection. Using natural plant extracts and oils as antifungal agents might be a viable solution to this issue (Tabassum *et al.,* 2014). Several plants, including *Bucida buceras* (black olive tree), *Breonadia salicina, Harpephyllum caffrum, Olinia ventosa, Vangueria infausta* and *Pinus gerardiana* have been investigated for their antifungal properties (Mahlo *et al.,* 2016). Amongst these medicinal plants, the *Pinus gerardiana,* locally known as (Chilgoza), are taxonomically the type of three species in *Pinus* subsection *Gerardiana*, the Asian nut pines. Syn. *Pinus gerardii*. The trees are mostly (10-25m) tall, ecologically distributed and grown wildly at the high altitudes of the mountains. Suleiman Mountain Range is an extension of the Hindu Kush. It lies at the junction of provincial borders of Balochistan and Khyber-Pakhtunkhwa, Zhob, and Sherani Districts of Balochistan province in north Pakistani Himalia range, eastern Afghanistan, Jammu-Kashmir, Tibet China, and an adjacent mountain range of Himalia in Indian territory. The seeds (pine nuts) are 18-24 mm long and 4-7 mm broad, with a thin shell and a rudimentary wing. The seeds are not shed but are retained by the wing adhering. The trees resist the climate having temperature ranging from -17 to 42°C. Some samples of the trees reported having age of approximately 300 year (Thomas *et al.,* 2003). Various formulations and dosage forms are being developed every year from these medicinal plants. These medicinal extracts were mostly used as oral, inhaler and topical. In which the transdermal drug delivery was found to have very significant role in

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the designing of different dosage forms and uses of natural drugs for effectiveness by local population as well the Nutraceutical industries in different preparations (Destaillats *et al.,* 2010). Amongst all the transdermal delivery system is also very important and considered to be safe route for administrating the drugs from skin to systemic circulation to get more effect and safe (Delgado *et al.,* 2002). In skin diseases like microbial infections and fungal infections (Krauze *et al.,* 2002). different plants and their parts are being started practicing but very little work regarding herbal extract of indigenous plants of Balochistan through transdermal drug delivery system have been scientifically reported so far (Maghraby *et al.,* 2006).

MATERIALS AND METHODS

Plant selection and collection

The plant was collected from the mountainous area of the Sharani district, Balochistan, Pakistan. The plant's identification was made by the Botany Department, University of Balochistan, Quetta, Pakistan. The evergreen leaves (needles) of the tree, which were well-nourished, mature and healthy, were carefully removed from the branches, exposed to sunlight, filled in a cotton bag, and brought to the lab.

Chemicals and equipment

Methanol, acetonitrile, n-hexane, ethyl alcohol, chloroform, acetone, disodium hydrogen phosphate, potassium hydrogen phosphate (Merck Germany), Hard paraffin, wool fat, absolute ethanol, soft white paraffin, diethyl ether, die chloromethane, chloroform, diethyl acetate was purchased from Sigma-Aldrich (oslov, Norway). Glacial acetic acid and HPLC grade water was prepared by Mille-Q system (Millipore, Milford, MA, USA). Chemicals and equipments were provided by the University of Balochistan and Department of Pharmaceutics, Faculty of Pharmacy. The Islamia University of Bahawalpur, Pakistan.

Extraction and filtration process

Plant needles were well cleaned and dust was removed from all parts and soaked as per the official method in different solvents. With the help of filter paper (Whatman's), the soaked materials were filtered accordingly. A rotary vacuum evaporator was used for extraction; this extract was evaporated under 42°C at reduced pressure. The evaporation continued until the liquid material was converted to a semisolid gum-type substance. The obtained extract was collected in a wide-mouth glass container and stored in the refrigerator at O°C to prevent microbial contamination

Analysis of essential oils by GC-MS

Essential oil components were analyzed using Thermo-Finnigan SSQ 7000 quadrupole and Shimadzu GC-MS-QP 2010 (Kyoto, Japan). Restek, Bellefonte, PA, USA, provided the capillary column (Rtx-5MS, 30m, 0.25mm internal diameter, 0.25m film thickness). After 2 minutes at 45°C, the temperature escalated linearly to 300°C over 5 minutes, 250°C injector and detector. Before testing, samples were diluted with 1% n-hexane. Automated 1 L/1.15 sample injection and Helium carried 1.41 mL/min. Mass spectrometer parameters: 70 eV ionization voltage, 200C ion source temperature and 35 to 500 AMU scan range. From mass spectra and retention indices (RI), essential oil components were determined. Comparing NIST-11, Wiley Mass Spectral Database and published data (Gad *et al.,* 2021).

Ointment preparation

Simple ointment 50.0g was prepared by melting hard paraffin 2.35g at 60°C, beeswax 2.35g was added at the same temperature and 2.35g of Cetostearyl alcohol. The mixture was allowed to cool with continuous stirring until it reached a temperature of 30-35°C. After that, white paraffin of 40.35g and extract of 2.5g (P. gerardiana) were added, allowing the ointment to cool at room temperature of 25°C (Gul *et al*., 2019). Table 1.

Determining antifungal activity

While using the poisoned food technique, the antifungal activity of test materials was tested against Bipolaris specifera, Alternaria alternata, Curvularia lunata. The plant extract's essential oil was sterilized in 9cm potato dextrose agar. Plant extracts were poured on fungus-cultured Petri dishes. They were then incubated at 26°C. Growth in control Petri dishes was measured every 24 hours. Antifungal activity (% age inhibition) = (Da - Db / Da) x 100. (MALAKA *et al., 2021*)

Conductivity and pH measurements

The Formulation (Ointment) was designed and prepared according to the pH value of normal human skin pH 4.85 + 0.5 and formulations were kept under different conditions for digital pH- Meter determination. The formulation's electrical conductivity (σ) was tested using WTW Cond 197i (Weilhein, Germany). The experiment was revised three times and the readings were calculated (Gul *et al.,* 2018).

Rheological studies

Viscosity Brookfield RVDV ultra programmed Rheometer (Brookfield Engineering Labs Middle-boro, MA) with spindle CP41 was selected to approximation viscosity of the ointment in triplicate by rotating the spindle at 10 cpm at 25°C. The interpretations were recorded as triplicate and mean was calaculated (Yousaf *et al*., 2020)

In-vitro study protocol

Franz diffusion cell equipment (PermeGear, Hellertown, PA, USA) was utilized for *in vitro* studies of *Pinus gerardiana* extract. The receptor medium and membrane

selected on the basis of its compatibility of drug and ointment formulation. Receptor media was consist of phosphate buffer and ethyl alcohol (75:25). The receptor solution was filtered via 0.2-μm membrane, selected temperature of the solution was kept at 32°C in a circulating temperature-controlled bath set to 32.5°C. Equipment Franz diffusion cells must be free from air bubbles. It was stabilized for 30 minutes before use.

Sampling from each cell of the Franz diffusion cell, were drawn 1 ml while using a 1-ml syringe via the sampling point. During the time of sampling, stirring not using, after sampling stirring was continued with fresh medium. During the whole time of the sampling process, we assured no bubbles in the receptor solution or in the syringe which affect the volume and permeability. Sampling intervals were at 0.5, 1, 2, 3, 4, 5, 6 and 12 hours and received samples were assessed by HPLC (Gul *et al*.,2019).

Pharmacokinetic Studies of *Pinus gerardiana* across the cellulose membrane. The amount of drug in the receptor solution was measured (0-12h) by HPLC and the released quantity of drug was assessed and computed. Linear regression analysis and parameters of drug release for each equation were calculated. The correlation coefficient (R2) was analyzed for each equation by each kinetic equation to ameasure whether the release permeation of the drug via the membrane follows a zero order, first order, Higuchi, Korsmeyer-Peppas, or Hixon-Crowell model. All calculations were conducted with the following kinetics equations software, DD Solver (a Microsoft Excel 2007 add-in program) (Gul *et al*., 2019).

Stability Studies

Ointment formulated samples were looked for stability as per ICH guide line at different temperature. All the formulations were checked for the color, odor, consistency, viscosity, pH and conductivity for the time of three months (Gul *et al.,* 2019).

STATISTICAL ANALYSIS

SPSS version 21, (IBM, USA) software was used to analyze formulation (Gul *et al.,* 2018). Kinetics equations using DD Solver (a Microsoft Excel 2007 add-in program) (Yousaf *et al.,* 2020).

Table 1: Quantities of ingredients during Ointment preparation

S. No	Plant Extract	Hard paraffin	Extracted Drug	Bees Wax	White Paraffin	Cetostearyl alcohol
1	*Pinus gerardiana*	2.35 g	2.5 g	2.35 g	40.35g	2.35g

Table 2: Chemical composition of the essential oil*s from the needles of Pinus gerardiana*.

S. No	Essential oil Found	Chemical Nature	Yield Percentage (%)
1	Alpha pinene	Monoterpene	26.68
2	Camphene	Monoterpene	0.41
3	Beta-pinene	Monoterpene	58
4	+Beta pinene	Monoterpene	2.6
5	Para cymene	Monoterpene	0.22
6	Limonene	Monoterpene	1.07
7	Beta-phellandrene	Monoterpene	0.28
8	Linalool	Oxygenated Monoterpene	0.11
9	Alpha- pinene oxide	Oxygenated Monoterpene	0.2
10	Cis-sabinol	Oxygenated Monoterpene	0.11
11	Trans-pinocarveol	Oxygenated Monoterpene	0.96
12	Verbenol	Oxygenated Monoterpene	0.74
13	Pinocarvone	Oxygenated Monoterpene	0.88
14	4-Terpineol	Oxygenated Monoterpene	0.05
15	Alpha-Terpineol	Oxygenated Monoterpene	2.05
16	Myrtenal	Oxygenated Monoterpene	3.35
17	1-Verbenone	Oxygenated Monoterpene	0.11
18	Bornyl acetate	Oxygenated Monoterpene	0.19
19	Copaene	Sesquiterpene	0.23
20	Alpha-murolene	Sesquiterpene	0.15
21	Alpha-bisabolene	Sesquiterpene	0.16
22	Caryophyllene oxide	Sesquiterpene	0.55
23	Beta bisabolene	Sesquiterpene	0.53
24	Isoledene	Sesquiterpene	0.1

Table 3: Antifungal activity *Pinus gerardiana* (% age inhibition) = (Da - Db / Da) x 100

S. No	Name of organism	Zone of Inhibition
01	*Bipolaris specifera*	2.98±0.1 µg/ml
02	*Alternaria alternate*	3.48±0.21 µg/ml
03	*Curvularia lunata*	5.04±0.24 µg/ml

Experiment was conducted in triplicate and the IC_{50} values were given as mean ±SD;

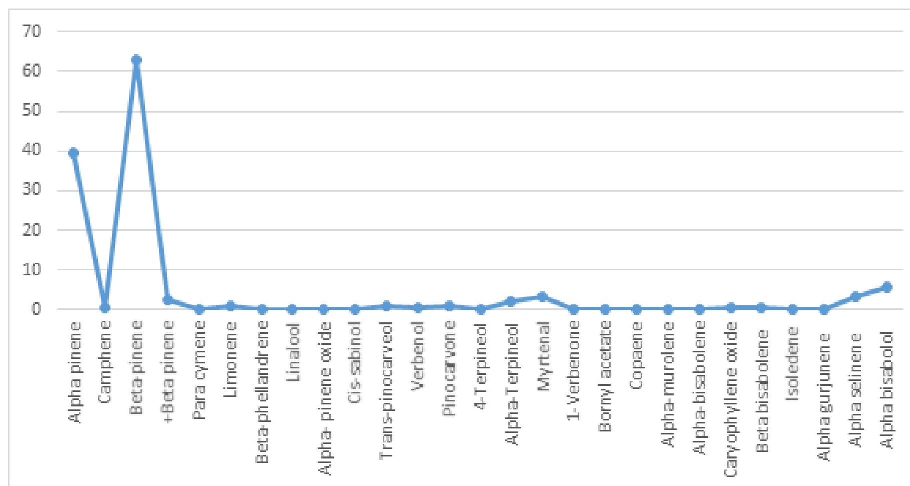

Fig. 1: percentage of the essential oil*s from the needles of Pinus gerardiana*.

RESULTS

Phytochemical screening

Leaf extracts were GCMS-analyzed to identify the chemical composition of *Pinus gerardiana* essential oil. About 27 components were obtained, and the analysis duration was 15-55 minutes. Out of 100% of the extracted essential oil of Pinus gerardiana, the identified compounds were -pinene (26.68%), -pinene (58%), Myrtenal (3.35%), Terpineol (2.05%), and Limonene (1.07%). The essential oil included seven monoterpene compounds (89.04%), eleven oxygenated monoterpenes (8.75%), and only (2.21%) sesquiterpene compounds (Copaiene, Isoledene, Beta-Caryophyllene, Alpha-murolene, Alpha-bisabolene, and Caryophyllene oxide). Monoterpenoids dominate essential oil composition, as shown in table 2 and fig. 1. In previous studies monoterpenes and sesquiterpene hydrocarbons were identified as the primary components of the essential oils of numerous Pinus species (Mitić *et al.,* 2018).

Antifungal activity

Pinus gerardiana oil showed antifungal efficacy against three harmful fungi (Alternaria alternata, Curvularia lunata, and Bipolaris specifera). Bipolaris specifera's zone of inhibition was 2.980.1g/ml, Alternaria alternative was 3.480.21g/ml and Curvularia lunata was 5.040.24g/ml, shown in table 3. Anti-fungal activity of *Pinus* plant species has been reported by different studies in Pakistan (Iqbal *et al.,* 2011) and other countries (Sharma *et al.,* 2018).

A similar study of *pinus gerardiana* plant extracts reported prominent antifungal activity at different doses 1000mg zone of inhibition 12.15±0.5 and 1500mg zone inhibition 15.01±0.5 (Sharma *et al.,* 2015). These findings have been supported by the chemical constituents of the plant containing Monoterpene, Oxygenated Monoterpene and Sesquiterpene, as shown in table 3. However, other studies have reported on pine nuts' phytochemical composition, all revealing strong antioxidant potential, being their concentration higher than that in phenolic compounds (Hoon *et al.,* 2015).

Physiochemical evaluation of ointments formulation

The extract of *Pinus gerardiana* containing ointment was tested for physicochemical analysis and the pH =5.9 was recorded within the normal range of skin pH (5.4-5.9), and similar results were described by other studies. The conductivity =0.1 and viscosity = 22.24± 0.09 were in the normal range, as shown in table 5.

Stability

The stability was checked in different storage conditions at different temperatures, freezing -2C°, 8C°, 25C° and 40C°. The storage condition was found to be different in colors and odors at different temperatures. The viscosity and pH were found to be no change.

The stability was checked for three months and the containers were closed tightly for the preservation of any contamination.

Table 4: Antifungal activity of *Pinus gerardiana* extracts against *Curvularia lunata, Alternaria Alternata and Bipolaris specifera.*

Percentage growth inhibition of *Curvularia lunata*				
Concentration	PGO (%)	PGLC (%)	PGLM (%)	PGLA (%)
1mg/ml	18	0	0	0
2mg/ml	25	0	0	0
3mg/ml	34	0	09	0
4mg/ml	41.7	12	14	0
(IC_{50}) mg/ml	5.04±0.24	NA	1	NA
Percentage growth inhibition of *Alternaria Alternata*				
1mg/ml	19	13	15	11
2mg/ml	18	28	24	24
3mg/ml	43	11	39	10
4mg/ml	57	35	36	14
(IC_{50}) mg/ml	3.48±0.21	5.9±0.26	6.2±0.32	NA
Percentage growth inhibition of *Bipolaris specifera*				
1mg/ml	29	0	12	0
2mg/ml	40	07	17	0
3mg/ml	49.5	12	28	0
4mg/ml	61	17	36	0
(IC_{50}) mg/ml	2.98±0.1	1	5.7±0.23	0

IC: inhibitory concentration, mg: milli gram, ml: milli liter, PGO: *Pinus gerardiana* essential oil, PGLC: *P. gerardiana* leaf chloroform extract, PGLM; *P. gerardiana* leaf methanolic extract, PGLA: *P. gerardiana* leaf aqueous extract

Table 5: pH, viscosity and conductivity of *Pinus gerardiana*

S. No	Parameters	Inference
1	Color	White
2	Odor	Characteristics
3	Consistency	Soft semi solid
4	pH	5.9
5	Conductivity	0.1
6	Viscosity	22.24± 0.09

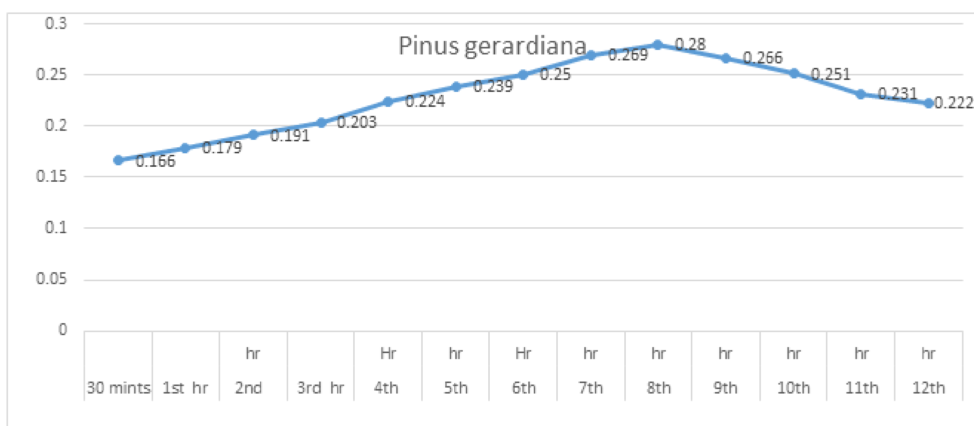

Fig. 2: *In vitro* release of *Pinus gerardiana* from an ointment

In vitro release of anointment

Pinus gerardiana ointment sample was carried out through Franz diffusion cells by placing 1g ointment sample into the open cap having an artificial cellulose membrane fixed on the Franz cell.

DISCUSSION

Pinus gerardiana leaf (needle) essential oil (PGO) showed considerable antifungal activity against *Curvularia lunata, Alternaria alternative* and *Bipolaris*

specifera with IC50 values of 5.040.24mg/ml, 3.48+0.21mg/ml and 2.98+ mg/ml respectively. *Curvularia lunata* and *Bipolaris specifera* were not inhibited by *P. gerardiana* leaf chloroform extract (PGLC), while *Alternaria alternata* was with IC50 values of 5.70.23 mg/ml shown in table 3. PGLM demonstrated weaker inhibitory action against *Curvularia lunata* than against Alternaria alternative and *Bipolaris specifera* (IC50: 6.20.32 and 5.70.23) table 4 fungi were not inhibited by *P. gerardiana* leaf aqueous extract (PGLA).

The released quantity of extract was determined at different time intervals first after 30 minutes, followed by 1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 7hrs, 8hrs, 9hrs, 10hrs, 11hrs and the final sample was taken after 12th hour of the experiment was started accordingly and release quantity of ointment was determined at different time intervals (from 30mins to 12 hours) ranged from 0.166 -0.280 as shown in table 6 and fig. 2. The drug release kinetics follows, Korsmeyer-Peppas model.

CONCLUSION

Pinus gerardiana ointment was successfully formulated. It was tested for antifungal physicochemical activities, showed significant antifungal activities on different pathogens; the plant extract showed easy penetration through artificial membrane. So it was considered to be more beneficent via transdermal route. further studies are necessary to be carried out the efficacy of *Pinus gerardiana* ointment in vivo.

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